AD_____

BINDING ASSAYS FOR THE QUANTITATIVE DETECTION OF P. BREVIS POLYETHER NEUROTOXINS IN BIOLOGICAL SAMPLES AND ANTIBODIES AS THERAPEUTIC AIDS FOR POLYETHER MARINE INTOXICATION

ANNUAL REPORT

Daniel G. Baden

15 December 1988

Supported by
U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-87-C-7001

University of Miami
Coral Gables, Florida 33149-1098

SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION: unclassified	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT: Approved for public release; distribution unlimited
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION: University of Miami; 6b. OFFICE SYMBOL (If applicable):	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code): Coral Gables, FI 33149-1098	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING/SPONSORING ORGANIZATION: U.S. Army Medical Res. and Develop. Command; 8b. OFFICE SYMBOL (If applicable):	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: DAMD17-87-C-7001
8c. ADDRESS (City, State, and ZIP Code): Fort Detrick, Frederick, MD 21701-5012	10. SOURCE OF FUNDING NUMBERS: PROGRAM ELEMENT NO. 62770A; PROJECT NO. 3M1 62770A871; TASK NO. AA; WORK UNIT ACCESSION NO. 402

11. TITLE (Include Security Classification)
Binding Assays for the Quantitative Detection of P. brevis Polyether Neurotoxins in Biological Samples and Antibodies as Therapeutic Aids for Polyether Marine Intoxication

12. PERSONAL AUTHOR(S)
Daniel G. Baden

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual	FROM 12/1/87 TO 11/30/88	15 December 1988	29

16. SUPPLEMENTARY NOTATION

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	Keywords: marine toxins, antibodies, sodium channels, receptor binding, brevetoxin, RA1
06	01		
06	15		

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

The polyether lipid-soluble toxins isolated from the marine dinoflagellate Ptychodiscus brevis (formerly Gymnodinium breve) can be detected using two separate types of specific binding reaction. Using tritiated PbTx-3 as a specific probe for binding to voltage-dependent sodium channels in rat brain synaptosomes or to specific polyclonal antibodies, binding equilibria and displacement by unlabeled brevetoxins have been compared. Labeled toxin can be displaced in a competitive manner by any of the other 5 naturally-occurring toxins; the quantitative displacement ability of each appears to reflect individual potency in fish bioassay. A comparison of ED50 in Radioimmunoassay and ED50 in synaptosome binding assay indicates that the former assay is useful for detection of toxins which possess the structural backbone of PbTx-3, the immunizing hapten. Thus, the two assays have quantitative applicability; the sodium channel with respect to potency and the antibodies with respect to structure. Microtiter plate assays utilizing each specific brevetoxin binding

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT ☐ DTIC USERS	unclassified
22a. NAME OF RESPONSIBLE INDIVIDUAL: Mary Ellen Bozeman	22b. TELEPHONE (Include Area Code): 301-663-7325
	22c. OFFICE SYMBOL: SGRD-RMI-S

DD Form 1473, JUN 86 — Previous editions are obsolete. — SECURITY CLASSIFICATION OF THIS PAGE

A

19 (cont)

component and enzyme-linked toxin hapten have been successful and indicate a general applicability of colorimetric prototypes. There, is however, considerable manipulation required to decrease non-specific binding of the hydrophobic toxin-enzyme complex to the plates. Preliminary studies aimed at producing monoclonal antibodies have been explored using brevetoxins linked to keyhole limpet hemocyanin (KLH). Purification of the specific binding components have been attempted using toxin affinity column, molecular size partitioning chromatography and Protein G-Sepharose columns.

Foreword

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigator(s) haved adhered to the "Guide for the Care and Use of Laboratory Animals", prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHHS Publication No. (NIH) 86-23, revised 1985).

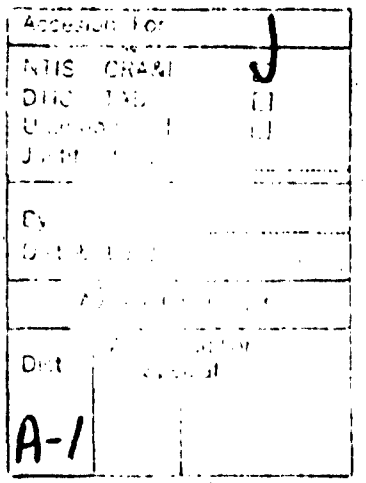


TABLE OF CONTENTS

I. List of Appendices, Illustrations, Tables

II. Statement of the Problem

This contract is concerned with the development of diagnostic methods and therapy for exposure to polyether toxins produced by marine dinoflagellates. We have proposed two separate binding assays which have potential value in the quantitative detection of these toxins in biological samples. Our specific aims are to:

(1) develop and refine *in vitro* radiometric binding assays to detect polyether marine neurotoxins in biological samples using tritiated brevetoxin PbTx-3 (formerly T17) as radiometric probe and employing antibodies prepared in a goat against toxic component PbTx-3 produced by laboratory culture of *Ptychodiscus brevis* or synaptosomes prepared from rat brain;

(2) determine the sensitivity and specificity of the binding assays using brevetoxin standards mixed with biological samples of clinically-obtainable types, i.e.serum, mucousal secretions, urine and or feces;

(3) using goat antibodies or solubilized brevetoxin binding component from rat brain, develop enzyme-linked assays to further simplify the procedure for routine use;

(4) examine potential cross-reactivity of the binding assays with respect to other polyether toxins, and hence their usefulness in the detection of other lipid-soluble marine polyether toxins;

(5) examine the feasibility of using available antibodies as therapeutic agents, first using competitive *in vitro* molecular pharmacological binding assays, and later by examining the reversal of toxic effects in animals by immunoassay;

(6) provide reagents adequate for 10,000 assays, including radioactive toxin probe, and data on tests and evaluations. Detailed protocols will accompany reagents.

III. Background

A. Toxins

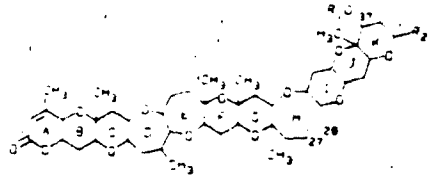
We routinely isolate six brevetoxins from laboratory cultures of *P. brevis*, all based on the two polyether backbones (1). In logarithmic cells, the two predominant toxins are PbTx-1 and PbTx-2 (see Figure 1). In stationary cells, approximately the same relative amounts of PbTx-1 and PbTx-2 are present on a per cell basis, but now in addition PbTx-3, PbTx-5, PbTx-6 (based on the backbone present in PbTx-2), and PbTx-7 (based on the backbone present in PbTx-1) appear. Two additional synthetic toxins, PbTx-9 and PbTx-10, are available by chemical reduction (2).

B. Molecular Pharmacology

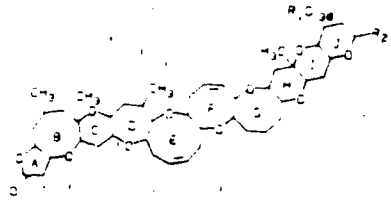
We have previously shown that PbTx-3 binds to site 5 associated with voltage-sensitive sodium channels, and have determined a K_D of 2.9 nM and a B_{max} of approximately 7 picomoles/mg synaptosomal protein (see Figure 2) for this site. We also demonstrated that tritiated PbTx-3 could be displaced in a specific manner from its binding site by either natural or synthetic brevetoxins. Our initial observation was that

displacement efficiency was linked in a positive fashion with potency in animals. Specific displacement curves correlated well with the potency of each individual purified toxin. Differential lipid solubility of each of the natural brevetoxins made it imperative to include Emulphor EL-620 in all experimental tubes.

In addition to developing displacement curves for the six toxins (n=2), we had sufficient toxin material for PbTx-1,-2,-3, and -7 to calculate K_is. These are summarized for several species in Table 1.



Type-1



Type-2

Toxin	Type	R_1	R_2
PbTx-1	2	H	
PbTx-2	1	H	
PbTx-3	1	H	
PbTx-5	1		
PbTx-6	1	H	 27, 28 epoxide
PbTx-7	2	H	
PbTx-8	1	H	
PbTx-9	1	H	
PbTx-10	2	H	

Figure 1. Structures of the Brevetoxins.

Table I. Inhibition Constants for Derivative Brevetoxins Derived from the Cheng-Prusoff* Equation

Toxin	K_i (nM)		
	Turtle	Fish	Rat
PbTx-1	0.39	10.10	0.72
PbTx-2	1.34	23.57	3.51
PbTx-3	1.96	37.04	2.47
PbTx-5	----	----	2.68
PbTx-6	----	----	6.60
PbTx-7	----	----	0.85

*see Reference (3).

C. Immunology

At a time when only the structures of PbTx-2 and PbTx-3 were known, we began developing immunoassays for the detection of brevetoxins in marine food sources (4). Utilizing bovine serum albumin-linked brevetoxin PbTx-3 as complete antigen, we succeeded in producing antiserum in a goat. We chose goats for the large quantities of immune serum which we could obtain, provided we could raise an antibody population. Subsequent characterization of the immune serum obtained indicated that both PbTx-2 and PbTx-3 were detected in approximately equivalent manners. Although oxidized PbTx-2 was not potent in either fish or mouse bioassay (5), it did displace PbTx-3 in competitive radioimmunoassay, an indication that potency was not reflected in RIA.

With the description of new brevetoxins based on the PbTx-2,PbTx-3 -type structural backbone (1), it was of interest to examine the competitive abilities of these new toxins. Based on the types of structural derivatives in tthis toxin series, we felt that new information regarding the epitopic sites on the brevetoxin backbone might be uncovered. In the same vein, the new structural backbone present in PbTx-1 and PbTx-7 might give us further insight into epitopic sites (the terminal 3 to 4 rings are identical) on brevetoxins.

Radioimmunoassay displacement curves indicated that the antibody recognizes and binds the toxins which possess the type of structure depicted on the left(type-1) of Figure 1 with much higher affinity than it does the toxins whose backbone is illustrated in Figure 1 on the right (type-2). This is not surprizing because the antibody was produced by immunization with BSA-linked PbTx-3, a type-1 toxin (18). Statistical analysis of ED_{50} values reveal that there are no statistical differences between the efficiencies with which PbTx-2, PbTx-3, and PbTx-5 displace tritiated PbTx-3 from the antibody-hapten complex (t-test, $p<0.1$). Analysis of 50% displacement values for PbTx-1 and PbTx-7 (both type-2) revealed no statistically-significant difference ($p<0.001$). With the exception of PbTx-6, a significant difference was consistently found, however, between the curves for the two toxin backbones. Type-1 toxins are approximately 10-fold more efficient than are type-2 toxins at displacing tritiated PbTx-3 from the binding site. The exceptional case, PbTx-6, is a 27,28 epoxide of

a type-1 toxin. An epitope on the toxin molecule may involve the configuration around the 27,28 carbon unsaturation (summarized in Table II) (6).

TABLE II. CORRELATION OF POTENCY WITH RADIOIMMUNOASSAY AND SYNAPTOSOME ASSAYS

Toxin	Synaptosome ED_{50} (nM)	K_i	LD_{50} (nM)	Radioimmunoassay ED_{50} (nM)
PbTx-1	3.5	7.1	4.4	93.0
PbTx-7	4.1	8.9	4.9	92.0
PbTx-2	17.0	16.1	21.8	22.0
PbTx-3	12.0	37.0	10.9	20.0
PbTx-5	13.0	----	42.5	10.1
PbTx-6	32.0	----	35.0	112.0

ED_{50} are defined as the toxin conc at which 50% displacement of tritiated PbTx-3 from sodium channels or antibody occurs. LD_{50} are determined by incubation of Gambusia affinis with toxin in 20 mL seawater for 60 minutes. K_i are determined as described in the text.

In addition, we began to explore methods for converting the RIA to an enzyme linked form. We sought to use an enzyme system which was stable, produced a color reaction which would be visible to the naked eye (even though our evaluation would take place in a microtitre plate reader), would lend itself to coupling enzyme to either toxin or antibody, and would possess an enzymatic activity that was absent in mammalian systems (to reduce background color reactions).

The basic assay under development followed a noncompetitive enzyme immunoassay sandwich technique (figure 2). Heterogeneous system assays (7,8) such as these may be performed as either competitive or noncompetitive types, and may be either enzyme-antibody labeled or enzyme-hapten (antigen) labeled. Thus, the greatest flexibility is gained employing such techniques, and many different variations may be developed to meet defined criteria.

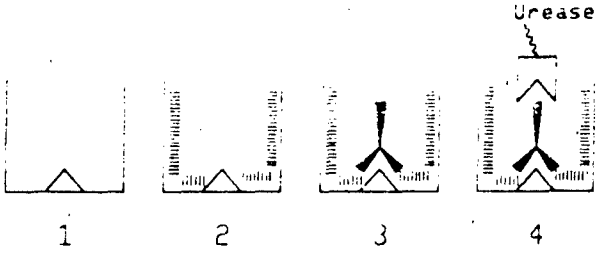


Figure 2. Non-Competitive Brevetoxin-Antibody-Protein A Urease ELISA.

In order for the proposed assay to work, toxin PbTx-2 (illustrated as triangles) had to be successfully bound to the microtitre plate wells. Unlike standard enzyme immunoassay procedures, where water-soluble IgG is adsorbed to the plastic plates, it was necessary to investigate the binding kinetics and equilibria of toxin binding. It is imperative that the solid phase should adsorb an adequate amount hapten in a reproducible manner, and that variability at this stage will affect the ultimate precision of the assay.

Binding of PbTx-2 (step 1 of figure 2) was evaluated in three media : ethanol, a solvent in which the toxins are reasonably soluble; phosphate buffered saline, in order to promote partitioning onto the hydrophobic polystyrene surface; and carbonate buffer of pH 9.6, which is routinely used to bind IgG to plates. Following binding, complete Protein A-urease sandwich assays (steps 2-4) were carried out. We illustrated that PBS is the most suitable medium for toxin incubation. We demonstrated the linearity of the assay with respect to reaction time, illustrating the lack of end-product inhibition of the urease system (9).

The stability of the toxin-antitoxin adsorbed on the microtitre plate (step 3), when stored in a dry atmosphere at room temperature, indicated a probable long shelf-life of the reagents . Stability curves were carried out for 2 months with no loss in activity. Regardless of the blocking agent (shown as a hatched line in steps 2,3, and 4) we used, however, substantial amounts of non-specific binding of Protein A- urease was observed. Thus, backgrounds were many times very high, contributing to a low degree of specific sensitivity. In addition, the enzyme urease, while exhibiting a high turnover number and relative insensitivity to temperature during incubation, was very sensitive to heavy metals and pH (as well as the indicator dye, which is pH sensitive). Modifications to the ELISA technique were explored this contract year, investigating (i) toxin-enzyme conjugates, (ii) commercial enzyme-anti IgG conjugates, various blocking agents for the microtitre plates, a different enzyme and substrate for visualization, and monoclonal antibrevetoxin antibodies.

IV. Technical Approach

A. Synaptosome Binding Assay

Biological Preparation. Synaptosomes were prepared fresh daily from rat brain using the techniques described by Dodd et al. (10). Synaptosome integrity was evaluated using electron microscopy, or by ^{22}Na influx experiments. To prepare lysed synaptosomal fragments, the synaptosomal pellet was resuspended in 5 mM sodium phosphate (pH 7.4) and incubated with occasional stirring for 30 min in an ice bath. Protein was measured on resuspended intact synaptosomes or lysed synaptosomes just prior to binding experiments using the technique described by Bradford (11).

Toxin probe preparation. Natural toxins were used as obtained, purifed from cultures. Synthetic tritiated PbTx-3 and unlabeled PbTx-3 were prepared by chemical reduction of PbTx-2 using sodium borotritiide or sodium borohydride, respectively. Toxin PbTx-7 was produced by identical chemical reduction of PbTx-1 using borohydride. Precursor

toxins were mixed with equimolar reductant, each present in saturated solution. Under stirring conditions, the reactants were mixed and allowed to react for 3.5 min, after which time excess acetone was added as sacrificial substrate (reduced to propanol). The solvent and propanol was evaporated, and the residue was redissolved in minimal acetone. Acetone-soluble material was thin-layer chromatographed on silica gel plates using ethyl acetate/petroleum ether 70/30 as solvent, followed by high pressure liquid chromatography using an isocratic elution (1.4 mL/min) solvent of 85% methanol/15% water and monitoring absorbance at 215 nm.

Tritiated toxin was quantified employing uv HPLC detector tracings and standard curves were developed using unlabeled toxin PbTx-3. Radioactivity was determined using liquid scintillation techniques and appropriate quenched tritium standards. HPLC-purified radioactive PbTx-3 has a specific activity of 10-15 Ci/mmole, or one-fourth the specific activity of the chemical reductant. Aliquots of tritiated toxin are stored under nitrogen atmosphere at -20°C in ethyl alcohol solution. Labeled toxin is stable for 4-6 months, repurification by HPLC being performed as necessary.

Other toxins. Other brevetoxins were used as purified from cultures. Potency of individual brevetoxins was measured using Gambusia fish bioassay (4,5).

Binding assays. Binding of tritiated toxin was measured using a rapid centrifugation technique (5). Binding assays were performed in a binding medium consisting of 50 mM HEPES (pH 7.4), 130 mM choline chloride, 5.5 mM glucose, 0.8 mM magnesium sulfate, 5.4 mM potassium chloride 1 mg/mL bovine serum albumin, and 0.01% Emulphor EL-620 as an emulsifier; the latter being necessary to solubilize the high concentration of unlabeled PbTx-3 used in measurement of nonspecific binding. Binding experiments were also conducted in a depolarizing medium consisting of 135 mM KCl, 5.5 mM glucose, 0.8 mM magnesium sulfate, 1 mg/mL bovine serum albumin in 50 mM HEPES (pH 7.4). Synaptosomes (40-80 ug total protein), suspended in 0.1 mL binding medium minus BSA were added to a reaction mixture containing tritiated PbTx-3 and other effectors in 0.9 mL binding medium in 1.5 mL polypropylene microcentrifuge tubes. After mixing and incubating at 4°C for 1 hour, samples were centrifuged at 15000 x g for 2 minutes. Supernatant solutions were aspirated and the pellets were rapidly washed with several drops of a wash medium (9). The pellets were then transferred to liquid scintillation minivials containing 3 mL scintillant and the bound radioactivity was estimated using liquid scintillation techniques. Nonspecific binding was measured in the presence of a saturating concentration of unlabeled PbTx-3 (10 uM) and was subtracted from total binding to yield specific binding. Free tritiated probe was determined by counting directly an aliquot of the supernatant solutions prior to aspiration.

B Radioimmunoassay

Antigen Construction. Toxin PbTx-2 was purified to HPLC homogeneity as described above and potency was confirmed using the [illegible] bioassay (4,5). The toxin was unlikely to be antigenic because of its molecular size, and thus it was necessary to couple as hapten to a suitable antigenic carrier, in these cases to

bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH). We utilized the aliphatic aldehyde functions present in PbTx-2 as the coupling site, principally because the aldehyde is located on the terminal portion of each molecule and thus the toxin's spatial exposure during immunization would be enhanced.

Homogeneous toxin PbTx-2 was added to acetonitrile to yield a final concentration of 7 mg/mL. To this solution was added (in 3 equal increments at one minute intervals) sodium borohydride (as a saturated solution in acetonitrile). The final acetonitrile stoichiometry added was on the order of one mole reducing equivalents per mole toxin. For assessment of reducing efficiency, and as a tracer for later coupling steps, one uCi tritium labeled borohydride was added to the reaction mixture. Following reaction for 6 minutes under conditions of constant stirring, excess borohydride was degraded by the addition of one mL acetone (which is reduced to propanol). The resulting solution was thin-layer chromatographed on silica gel and ultimately was purified to homogeneity using HPLC. For purposes of our later coupling, we sought to utilize only the PbTx-3 reduction product. However, we wish to point out that PbTx-9, which corresponds to a doubly reduced product, also possesses a primary alcohol and could be used for coupling as well.

Purified PbTx-3 (reduced PbTx-2) was dissolved in a minimal volume of redistilled pyridine, and a ten-fold molar excess of succinic anhydride in pyridine was added with stirring. The reaction vial was sealed, and was heated to 85°C and stirred for 2 hours in an oil bath. Following reaction, each solution was dried under a stream of nitrogen, redissolved in minimal methanol and was chromatographedon silica gel in 70/30 ethyl acetate/ petroleum ether. Portions of each plate were sprayed with bromcresol green solution for detection of acids, and 1 cm portions of each plate were scraped and assayed for radioactivity by liquid scintillation techniques. Fractions which produced both acid-positive reaction (succinic acid) and radioactivity (toxin) were scraped, eluted, and weighed.

The free carboxyl function on each toxin-succinate derivative was covalently coupled to the ε-amino group of lysine resides in BSA or KLH by use of standard techniques we have employed previously (4). The procedure used was that employed for the covalent modification of steroid hormone when coupled to protein carriers (12), except that the final condensation step was lengthened to 12 hours. Following coupling, the mixture was dialyzed against 0.1 M sodium phosphate buffer (pH 7.4) for 24 hours, and 12 hours against phosphate-buffered saline (PBS). Antigen concentration was adjusted to 1 mg/ml toxin equivalents for immunization.

Immunization. A single female goat was immunized with 0.75-1.0 mg toxin equivalents of each complete PbTx-3 antigen at 2 week intervals for eight weeks, the first immunization containing complete Freund's adjuvant, and subsequent boosts being suspended in incomplete Freund's adjuvant. Thereafter, boosting proceeded at 14 day intervals. Serum was obtained just prior to the third immunization (six weeks), and thereafter on alternate weeks to each boost, for evaluation of serum titre. After the ninth immunization (approximately 20 weeks), two additional animals were introduced into the immunization protocol. These animals were trebled for baseline serum titres, and then were immunized as described above. These two animals are currently being

maintained, the first being boosted with BSA-toxin and the second being boosted with KLH-toxin..

Preparation of Antibodies. Each antiserum was treated with ammonium sulfate to yield a final salt concentration of 1.9 M. The mixture was stirred at 4°C for 1 hour and was then centrifuged at 12,000 x g at 4°C for 30 minutes. Precipitates were washed once with 1.9 M ammonium sulfate, dissolved in PBS, and dialyzed against PBS for 24 hours. The resulting crude antibody solutions were adjusted to 25 mg/ml protein for titre evaluation. Protein concentrations were measured according to the method of Bradford (11).

Evaluation of Titers in Serum Samples. For each bleeding, 30 uL aliquots of antibody preparation (0.75 mg protein) were added to duplicate tubes containing 0.5 mL PBS, and increasing amounts of [3H]PbTx-3 ranging from 2.0 to 2000 pg. A parallel null experiment was performed using pre-immune serum fractions, for evaluation of specific vet not brevetoxin antibody-specific binding. Non-specific binding in each case was determined in the presence of 10 uM unlabeled PbTx-3. As in the case with molecular pharmacological binding experiments, specific binding is defined as the difference between total and non-specific binding, i.e. it is a calculated value. The difference between specific binding values in pre- and post-immunization sera is defined as a measure of specific antibody induction. Ideally, pre-immune serum should exhibit only non-specific binding of tritiated brevetoxin. Incubation times and assay protocol are given below.

Radioimmunoassay. The procedure utilized was that described by Bogucki et al. (13) for the quantitation of serum digoxigenin levels in plasma. Aliquots of antibody solution (0.75-1.25 mg protein) were added to duplicate tubes containing 0.5 mL PBS, 2 ng [3H]PbTx-3, and known quantities of unlabeled toxin ranging from 0.6 to 200 ng. Duplicate control tubes were included, but without unlabeled toxin (total counts bound by antibody), or in the absence of antibody (total counts per tube). The incubation volumes were kept constant by the addition of PBS where needed.

Specific Assay Protocol. After incubation at 24°C for one hour and then overnight in the refrigerator, 0.5 ml of a suspension containing 2% charcoal (NIA grade) and 0.25% dextran in PBS was then added to each incubation tube except for those containing no antibody. Tubes were then mixed and incubated at 4° for five minutes, and then were centrifuged at 2000 x g for 5 minutes. Aliquots of 0.5 ml were placed in liquid scintillation mini-vials together with 3 ml liquid scintillant and counted against quenched standards in a Beckman LSC with an efficiency for tritium of 40%. Samples were counted for sufficient time to yield counting precision of 95%.

4. Microtiter Plate Assays

[illegible] assays were carried out in flat bottom 96-well polystyrene microtiter plates (Costar [illegible]) having a 0.3 mL capacity and a 6.4 mm diameter. Our work this year was concerned with development of [illegible] of the assays already enumerated, we made [illegible] and optimal assays this year. We also [illegible] investigate the use of rat brain synaptosomes in the

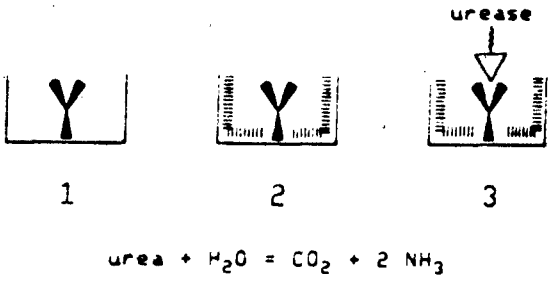
microtiter plate assays, once we had developed toxin-enzyme conjugates whic were active materials.

Immunoassays employing protein A-urease. The assay we developed last year (Figure 2) was a Protein-A-urease linked sandwich assay. We chose this for the following reasons: (1) protein A binds specifically to the Fc region of IgG and thus will minimize interference with Fab-toxin interactions; (2) protein-A interacts with most IgG Fc's, thus permitting its use for many antibodies created; (3) protein-A-urease is available commercially, thus assuring quality control; (4) urease has a high turn-over substrate rate and is not typically a mammalian enzyme, thus providing low background when examining biological fluids; (5) the assay is conveniently monitored using a dye-coupled (590 nm detection) reaction in response to released ammonium ion, allowing for microtitre plate monitoring colorimetrically.

That assay utilized initial binding of hydrophobic toxin to polystyrene (virtually quantitative) (step 1), followed by specific antibody binding (step 3), followed by washing. Protein A-urease was next added to bind specifically any toxin-specific antibody bound to adsorbed toxin (step 4). After a final wash, urea substrate solution containing bromcresol purple dye was added and the color reaction is evaluated photometrically.

PbTx-2 was used as hapten bound to the plate because of its higher hydrophobicity. Parameters such as optimal toxin concentration per well (in 200 uL PBS), optimal buffering solution for toxin adsorption, and optimum time and temperature for binding. Following toxin binding, brevetoxin specific antibody was added and parameters of time and temperature for binding, antibody concentration, and nonspecific protein blocking or lack of blocking were evaluated. Both of these perturbations were evaluated by classical "chequer board" arrays in the microtitre plates. Evaluation of stability of the microtitre plate-toxin-IgG complex in lyophilized state was evaluated for shelf-life. As indicated already, because of the unstable nature of the urease enzyme in Protein A coupled form, we abandoned Protein A-urease as a potential reagent for assays.

Enzyme immunoassays employing brevetoxin-urease conjugates. We sought to examine the stability, and efficacy of utilizing, brevetoxin covalently-linked to urease enzyme. The toxin-enzyme conjugate is constructed in an entirely analogous manner to the construction of complete antigen from brevetoxin PbTx-3 and protein. This is described on page 12. The only deviation from this procedure is that we utilized a limiting concentration of toxin-succinate in the coupling procedure to encourage a one-to-one stoichiometry of toxin to enzyme. This is effected by adding toxin-succinate conjugate to protein solution, rather than the converse of adding enzyme to toxin-succinate solution. The coupling stoichiometry again can be conveniently measured using a small quantity of tritiated PbTx-3 added to solution during coupling. The 1:1 stoichiometry is utilized based on the intuitive notion that (1) a minimal number of toxin molecules per enzyme molecule will lessen the likelihood of active site addition in the enzyme, and (2) that a greater toxin stoichiometry would increase the hydrophobic character of the conjugate, thereby increasing non-specific adsorption to the plastic plates. A schematic of the described protocol is illustrated in Figure 3.



$$urea + H_2O = CO_2 + 2\ NH_3$$

Figure 3. Microtiter Plate Assays Utilizing Brevetoxin-Urease Conjugates

Checkerboard experiments on 96-well plates were carried out using toxin-enzyme concentrations ranging in 2-fold dilutions from neat to 1/512; (2 picograms/well to 1.02 nanograms/well) and dilution of polyclonal antibodies ranging from neat to 1/1024 dilutions (0.75 mg to 0.7 μg/well). The initial step (step 1) involved antibody binding to the microtiter plate surfaces in the concentrations indicated. Adsorption buffer included 0.1 M Phosphate Buffered Saline (PBS, pH 7.4), 0.15 M Phosphate Buffered Saline (PBS, pH 7.2), and sodium carbonate buffer, pH 9.6. As potential blockers of non-specific binding sites (step 2), non-fat dry milk (Blotto), nonflavored gelatin at various concentrations (GelBlock), bovine serum albumin (BSA), and pre-immune serum (PIS) were utilized to minimize non-specific binding of toxin-urease conjugate in step 3. In step 3, the various dilutions of toxin urease were evaluated. The various incubation steps were carried out at room temperature, 37°C, and at 4°C. Appropriate blanks were evaluated, as well as the stability of the toxin-enzyme conjugate in neat form.

Enzyme immunoassays employing antibrevetoxin IgG-peroxidase conjugates. Brevetoxin specific antibodies were prepared as earlier described. Aliquots were specifically adsorbed to Protein G-Sepharose columns (Pharmacia) and washed to remove excess protein not of an IgG nature. IgG was then desorbed from the column using high ionic strength glycine buffer, and was concentrated by molecular sieve centrifugation (MW exclusion 10,000). Concentrated IgG was loaded on a brevetoxin affinity column produced by the carbodiimide condensation reaction between PbTx-3 succinate and Amino hexyl Sepharose (Pharmacia). (An identical procedure to that utilized for toxin coupling to protein was utilized to couple PbTx-3 succinate to the amino groups of the Sepharose derivative). Following specific adsorption of the brevetoxin antibodies on the column, unadsorbed material was washed free by PBS, pH 7.4. Specific brevetoxin antibody was washed from the column with 3 M NaCl and was desalted by dialysis. This material was utilized for coupling to peroxidase below.

Horse radish peroxidase (3 mg) was dissolved in 0.3 ml freshly prepared 100 mM $NaHCO_3$ in a small tube. Sodium periodate (8 mM, 0.3 ml) was added with stirring and the mix was allowed to react 2 hr at room temperature. Three ml of anti-brevetoxin goat IgG (about 2 mg protein) was desalted on a small column and was placed in 100 mM sodium

carbonate buffer, pH 9.2. The activated peroxidase and the IgG were rapidly mixed and pipetted into dry Sephadex G-25 where they are allowed to react for 3 hr at room temperature.

Following reaction, the conjugate was eluted from the Sephadex utilizing sodium carbonate buffer (pH 9.2), and was neutralized with freshly prepared $NaBH_4$ dissolved in 0.1M NaOH. After incubation at 4°C for 1 hr, protease inhibitors are added and the whole mix is diluted to twice volume with glycerol and stored at -20°C for later use.

For this particular assay, similar types of parameters were evaluated to ascertain optimum conditions. The protocol is illustrated in Figure 4 below. Initially, we sought to use unlinked toxin as initial adsorption step, as we did in Figure 1 for urease assays. However, it appears that this step is critical with hydrophobic materials such as these toxins and often they yield unpredictable results. Instead we utilized the keyhole limpet hemocyanin (KLH) toxin

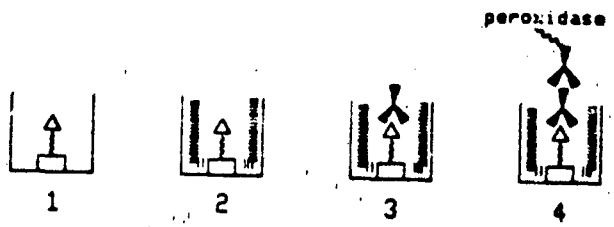


Figure 4. Enzyme immunoassays employing antibodies linked to peroxidase. The assays can utilize either brevetoxin-specific antibodies linked to peroxidase (Ab in step 3 would be linked), or anti-goat IgG to visualize brevetoxin specific antibodies adsorbed to toxin-KLH (as indicated in the figure).

conjugate as a primary adsorbant (Step 1). The material is quite hydrophobic, possesses an estimated 50-100 brevetoxin molecules per KLH molecule, and possesses characteristics exploitable of protein and the plastic plate interaction. In addition, the linker arm between the toxin and protein allows for a greater freedom of toxin-antibody interaction. Again, we evaluated many different KLH-toxin concentrations (step 1), the many different blockers (step 2) already enumerated, and several different washing and binding buffers. Commercial antigoat IgG-peroxidase conjugate (step 4) was utilized to evaluate the binding of brevetoxin specific antibodies to toxin-KLH adsorbed to the plate.

Enzyme immunoassays utilizing binding components derived from synaptosomes. The P3 fraction, resulting from the procedures employed for preparation of synaptosomes from rat brain (page 10), was utilized as biological preparation in identical dilutions as employed for antibody preparations described above. Standard checkerboard patterns were developed employing dilutions of each specific binding component, and toxin-enzyme conjugate. Dilutions of synaptosomes were incubated in microtitre plates for one hour, followed by brief washing with standard binding medium. Gelatin blocks followed for one hour, and were then rinsed with standard binding medium. Dilutions of toxin-urease conjugate were employed to acertain specific binding.

V. Results and Discussion

A. Synaptosomal Binding Assay

Species similarity of binding. Synaptosomes from rats, turtles, or fish were prepared to examine the binding characteristics of each with respect to brevetoxins. Table III outlines the results of the comparison, and illustrates that any of the three systems examined bind brevetoxins in a reproducible manner with approximately equal efficacy.

Table III. Comparison of Dissociation Constant (K_d) and Binding Maximum (B_{max}) in Fish, Turtles, and Rats*

Species	K_d (nM)	B_{max} (pMol/mg Protein)	Temp. Optimum (°C)	Specific Binding at K_d
Fish	6.1	1.45	23	80%
Turtle	1.5	2.25	4	80%
Rat	2.6	6.80	4	90%

*mean values for K_d and B_{max}, n=9,4,6 for fish, turtles, and rats respectively.

Specific binding of four tritiated brevetoxins in rat brain synaptosomes. A preliminary comparison of specific binding of tritiated PbTx-3, PbTx-7, PbTx-9, and PbTx-10 indicates an equivalent B_{max} and a progression of K_d values which parallel the relative potencies of the labeled brevetoxins. This is a further indication to us that binding affinity is the conservative requirement in the potency of the brevetoxins (Table IV), and further, that we may be able to utilize the toxins which are of higher specific activity for more detailed receptor characterization (14).

Table IV. Comparison of K_d and B_{max} for Four Different Tritiated Brevetoxin Probes in Rat Brain Synaptosomes

Toxin	K_d (nM)	B_{max} (pmoles/mg protein)
PbTx-3	2.13	6.99
PbTx-9	8.76	6.75
PbTx-7	1.91	6.38
PbTx-10	1.56	6.46

Our evidence indicates that, at a K_d concentration of tritiated PbTx-3, the $t_{1/2}$ for on- and off rates approximate 1-2 minutes. A closer approximation cannot be derived utilizing present protocols. There is no membrane potential dependence of brevetoxin binding to the high affinity, low capacity binding site known as Site 5. K_d = 2.6

(intact), 2.9 (lysed), 3.3 (depolarized) and B_{max} = 6.01 (intact), 5.83 (lysed) and 5.75 pmoles/mg protein (depolarized) (15).

Regardless of the organism used for synaptosomal preparations, it is apparent to us that the topographic characteristics of the brevetoxin binding site on the VSSC are comparable. Using brevetoxins PbTx-1-3, and PbTx-5-7, K_i data for specific displacement of tritiated PbTx-3 shows comparable data in each case (Table I in Introduction). The more hydrophobic type-2 brevetoxins are most efficacious in their ability to compete for site 5 binding (6).

Classes of brevetoxin binding sites. Two separate brevetoxin binding sites have been discovered in rat brain synaptosomes. The brevetoxins bind with an affinity constant which is consistently in the 1-5 nM concentration range, in good agreement with affinity data for other potent marine toxins like saxitoxin (16). In addition, the binding maximum in synaptosomes is also in good agreement with data for Site I toxins, which are known to bind to channels with a 1:1 stoiciometry. However, the allosteric modulation of sodium channel binding by other natural toxins by brevetoxins occurs at brevetoxin concentrations much higher, ca. 20-100 nM (17). This data is inconsistent with high affinity, low capacity binding.

Converse to this allosteric modulation which occurs at higher brevetoxin concentrations, is the finding that membrane depolarization, ^{22}Na influx and competitive displacement of tritiated brevetoxin binding by unlabeled competitors, is dose dependent in the same concentration ranges observed for the high affinity binding site (5). Thus, the allosteric modulation at other sodium channel binding sites appears to arise from brevetoxin interaction with a lower affinity, high capacity binding site.

Using classical Rosenthal analysis, we have been able to distinguish two separate specific brevetoxin sites (Table V).

Table V. The Two Brevetoxin Binding Sites

Site	K_d	B_{max}	Allosteric Modulator
5	2.6-3.3	5.7-6.8	No
_*	79.1-300.	63.7-180	Yes

*not numbered until further work can be accomplished.

The two site hypothesis is supported by brevetoxin inhibition constant data and double reciprocal cometition plots, which indicate a deviation from competitive tvpe patterns to non-competitive type patterns at higher competitor brevetoxin concentrations. The non-competitive displacement appears to be specific in nature, and is not likely due to changes in membrane fluidity. But, certainly more investigation is required before concrete conclusions can be offered.

B. Radioimmunoassay

The radioimmunassay was developed last year has been used this year almost entirely to give a baseline value to ELISA protocols, which have been a major thrust. Two potentially important developments have taken place this year, both involving a purification of brevetoxin specific antibodies for use both in RIA and ELISA. No further specific work has been undertaken with RIA, except that the KLH-PbTx-3 immunogen has been successful in eliciting antibody production in a goat. Titers are being evaluated weekly, with a biweekly immunization schedule. Currently, titers have exceeded BSA-PbTx-3 elicited titers, based on qualitative assessment and Ouchtelony plates employing whole antigen conjugate and specific sera.

Protein G-affinity columns. Goat serum was purified by loading 4 ml of a 10 mg/ml IgG solution onto a column constructed to contain 4 mg of PbTx-3 specifically bound. Single pass adsorpcion of the serum (6828 total units applied) resulted in 5254 Specific Binding Units (SBU) adsorbing and 1896 SBU passing through without adsorption, or a 73% specific adsorption of antibrevetoxin IgG to the column. Elution of the adsorbed IgG was accomplished using 3 column volumes of 0.15 M glycine-HCl buffer, pH 2.8., followed by desalting against PBS pH 7.4 ona commercial 10 ml desalting column (BioRad) (figure 5). Standard radioimmunoassays were performed on eluted fractions which were pooled to calculate specific binding units recovered.

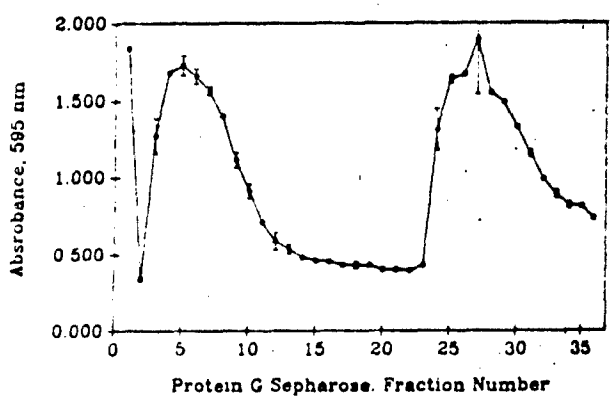


Figure 5. Protein G-Sepharose purification of brevetoxin specific antibodies. 4 ml of antibrevetoxin IgG was loaded on a 3 ml protein G-Sepharose column pre-equilibrated with PBS, pH 7.4. The sample was loaded as a single pass, measuring protein concentration in dropwise fractions. Following decreases in protein concentration to background values (fractions 15-20), the eluting buffer was changed from PBS to 0.15 M glycine (pH 2.8) to elute specifically bound IgG. Protein corresponding to eluted IgG elutes from fractions 23-40, fractions 25-35 being pooled as purified IgG. These pooled fractions correspond to 73% of specific binding units loaded on the column.

Brevetoxin affinity column. The brevetoxin affinity column has been utilized for IgG purification following Protein G-Sepharose separation. The pooled sample of IgG from the Protein G column (5254 SBU) was loaded on the Brevetoxin-Sepharose affinity column in minimal PBS, pH 7.4. Following single pass adsorption and elution with 3 M NaCl, 2210 SBU were recovered (7.2 mg protein), with a specific binding activity of 307 U/mg protein (figure 6). The peak not adsorbing to the column correpsonds to about 16.8 mg of protein (from a total of 24.4 mg loaded on the column) and possessed no specific binding activity. We feel therefore that the column is a useful tool for purification of IgG specific for brevetoxins.

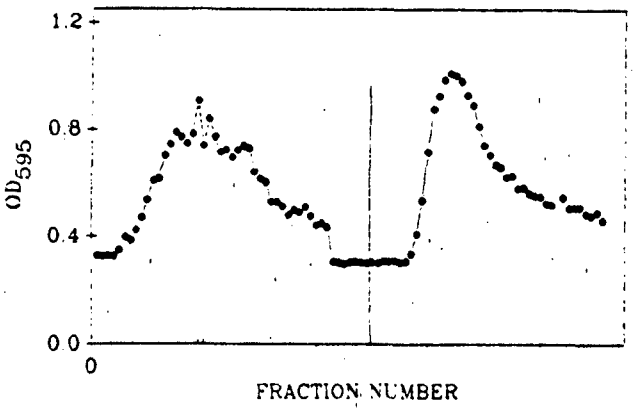


Figure 6. Adsorption and desortion of specific brevetoxin antibodies from a brevetoxin-Sepharose affinity column. A total of 5254 SBU (24.4 mg protein) of brevetoxin specific antibodies was loaded on brevetoxin-Sepharose affinity column prepared by carbodiimide condensation of PbTx-3 with aminohexyl Sepharose 4B. Following adsorption, the column was washed with 3 column volumes of PBS, pH 7.4 and the fractions were pooled. The first peak corresponds to 16.8 mg protein (and no specific binding units) which was not adsorbed. At the fraction indicated in the figure by the vertical line, the elution buffer was changed to 3 M NaCl and the peak which eluted was pooled. The peak corresponded to 2210 SBU and 7.2 mg total protein. The eluted peak was utilized for development of the microtiter plate assays described below.

C. Microtiter Plate Assays

Microtiter plate assays have been developed in five different ways, four utilizing antibodies and one utilizing synaptosomes. Each assay has distinct advantages and disadvantages. The high hydrophobicity of the toxins and their derivatives has been a principal difficulty in all our attempts at converting to enzyme-linked assays.

For most ELISAs, hydrophobicity can be exploited to "stick" antigen or antibody to the plate solid support. For brevetoxin microtiter plate assays, however, it was necessary to minimize nonspecific binding of toxin. Basic among our studies was the

opportunity to explore different methodologies for minimizing toxins, or toxin-conjugates, from non-specific adsorption to the plastic plates. Each assay will be outlined, identifying the finalized protocol, the problems encountered, the results obtained, and potential future work.

Protein A-urease assays (figure 2).

Protocol:

[1] incubate overnight at 4°C with PbTx-2 in PBS pH 7.4. toxin conc at 1 ng/well and 0.1 ml volume;
[2] aspirate and incubate 1 hour at 37°C with goat IgG at 0.75 mg/well in 0.1 ml volume.. Aspirate IgG solution;
[3] aspirate and block 1 hour at room temp. with 0.5% gelatin, 0.3 ml/well. Aspirate gelatin;
[4] add commercial protein A-urease at 10X excess theoretical IgG concentration in 0.3 ml volume. Incubate 1 hour at room temperature;
[5] aspirate urease conjugate and wash once with PBS (pH 7.4) and twice with distilled water;
[5] add urease substrate (consisting of per 100 ml in 0.1 mM sodium hydroxide: 8 mg bromocresol purple, 100 mg urea, 0.2 mM EDTA, all adjusted to pH 4.8) and monitor color development at 588 nm.

Results: Microtiter plates could be prepared as depicted in figure 2, and could be stored dessicated at either step three or four, i.e at the plate-toxin-IgG step or the plate-toxin-IgG-Protein A-urease step. Time course studies indicated no loss of enzyme activity under such conditions. The problems enumerated below preclude further development of the assay.

Problems:

[1] high non-specific binding even when blockers were used;
[2] low specific antibody binding to adsorbed toxin on plate;
[3] protein A urease unstable in refrigerator and lost activity;
[4] protein A binds to only 2 IgG subclasses form goats;
[5] competition assays using dilutions of brevetoxin were unsuccessful.

Future work: None. This assay appears to present too many difficulties to proceed further.

Toxin-urease assays (figure 3).

Protocol:

[1] incubate overnight with antibody solution (4 ng/well) in PBS, pH 7.4;
[2] aspirate and perform a 1 hour block;
[3] aspirate blocking agent and incubate with toxin-urease (range of 15 μg to 200 ng protein eq./well) for 2 hours at 37°C;
[4] aspirate and add urease reagent (as described above). Monitor at 590 nm.

Problems:

[1] high nonspecific binding of toxin-urease to plate;
[2] blocking agent tried to reduce nonspecific included 0.1-0.5% gelatin, 1% bovine serum albumin, each in the presence and absence of 0.01% tween;
[3] urease enzyme-toxin conjugate lost activity with time (over a 6-month period).

Results:

Successful ELISAs were carried out using a 1 hour 1% gelatin block. Immune sera from the goat and from mice begun on monoclonal immunization were detected quickly and with high sensitivity. ELISA results compared well with RIAs conducted on same serum. Competition assays using dilutions of cold PbTx-3 added at the same time as constant concentrations of toxin-urease gave successful results up to 10 nM cold toxin (figure 7).

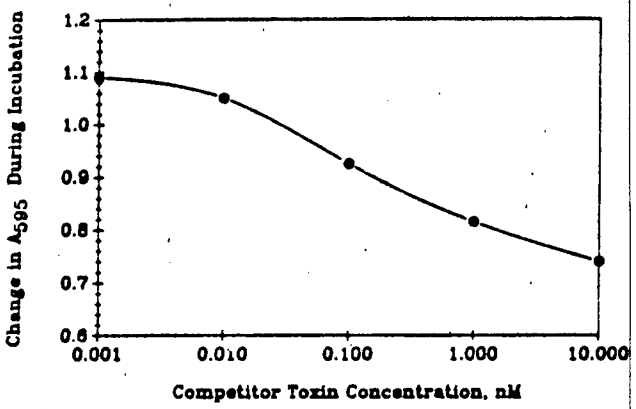


Figure 7. Toxin-urease assay conducted as presented in figure 3 schematic. Assays were conducted at room temperature, measuring change in absorbance at 595 nm using a microtiter plate reader. PbTx-3 concentrations ranged from 0.001 nM to 10 nm PbTx-3 IgG= 4 ng protein/well; toxin-urease= 8 μg protein/well.

Future Work:

We believe this assay utilizing derivatized brevetoxin has merit, clearly for its indication that enzyme-toxin conjugates retain enzyme activity and also because they are still recognized by brevetoxin specific antibodies. As described earlier, however, problems with stability and sensitivity of the urease enzyme preclude further work.

AntigoatIgG-antibody-peroxidase brevetoxin sandwich ELISA. (figure 4).(Used to optimize KLH-toxin binding to goat polyclonal antibody).

Protocol:

[1] Keyhole limpet hemocyanin-linked brevetoxin PbTx-3 (50 ng protein, approximately 0.2 ng PbTx-3/well) in 0.2 ml sodium carbonate buffer pH 9.6, was incubated overright at 4^{o}C;

[2] aspirate and add goat polyclonal antibody (mg to ng protein concentration ranges) to optimize Ab-Ag concentrations for 1 hour at room temp;
[3] aspirate and add commercial rabbit antigoat-horse radish peroxidase. Incubate 2 hours at room temp.;
[4] aspirate commercial preparation and wash plate with buffer;
[5] add commercial ABTS (2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) reagent and monitor development of color reaction.

Results:

[1] Strong positives and low blanks at up to $1:10^6$ goat antibody dilutions (7.5×10^{-7} mg/well) and $1:10^4$ rabbit antigoat (rag) peroxidase dilutions. 1:50 dilution of rag detects up to $1:10^6$ dilution goat Ab. 1:500 dilution of rag detects up to $1:10^5$.;

[2] Immulon 4 plates (Dynatech) appeared to give highest amount of specific KLH-toxin binding and lowest nonspecific binding of antibodies and antibody-enzyme conjugates;

[3] Preimmune goat serum as blocker gave best results for minimizing nonspecific binding of goat antobidy to plates, and also yielded the highest degree of specific reaction with KLH-toxin;

[4] Non fat dry milk was also an effective blocker.

Future Work:

The assay works well and is useful for evaluation of serum titers of antibody from animals being immunized. Our goat bleeds can be evaluated using this assay, employing each bleed serum in step 3 of the assay (figure 3) and employing the commerical rag antibody uniformly. This is the first assay we have developed of an ELISA format which appears to work reproducibly and which has practical applications. We believe that this is in part due to the time we have spent developing it, but further that the reagents and substrate (plates) were developed over many years to a totally reproducible state. Thus, the assays practical application should be developed further to capitalize on these aspects; i.e. the only variables now are KLH-toxin and goat (or other species as necessary) antibody, both of which are now routinely available in purified form.

Our future plans with this assay are to utilize the Protein G-sepharose column to pre-purify goat antibodies, and then follow with the toxin-sephadex affinity columns to further purify specific goat antitoxin antibodies, and to re-evaluate the yields at each step using the rag ELISA. We believe this assay will be a primary analytical tool for the laboratory.

<u>Goat antibrevetoxin antibody-peroxidase assay.</u> (figure 3, where antibody in step 3 is peroxidase linked).

Protocol:

[1] Goat polyclonal antibodies, pre-purified by sequential Protein G-Sepharose and PbTx-3-Sephadex affinity columns, were linked to horseradish peroxidase using a standard sodium meta-periodate method (18);
[2] incubate KLH-toxin conjugate in microtiter plates overnight at 4°C (dilutions from neat to 1:20000);

[3] aspirate and block 1 hour at room temp with nonfat dry milk;
[4] aspirate and incubate 2 hours at room temperature with antibrevetoxin-peroxidase (0.6 μg to 6.0 pg protein/well);
[5] aspirate and add ABTS substrate and monitor at 405 nm.

Results:

Strong signals were observed down to 1:20,000 dilution KLH-toxin concentrations, indicating that this reaction is both highly reproducible and optimized. However, full strength antibrevetoxin-peroxidase conjugate is required for the reaction. Two alternatives exist for this observation: (1) the conjugated peroxidase enzyme loses some acitivity upon brevetoxin covalent binding; or, (2) brevetoxin stoichiometries are not what we calculate.

Future Work:

The assay will be further refined to yield consistent results at high sensitivity. Conjugation procedure for toxin to peroxidase will be optimized including proper stoichiometry and enzyme activity evaluations with increasing toxin coupling. Some initial work, employing the following protocol, has been attempted:

Protocol:

[1] incubate overnight at 4°C with goat antibody in microtiter plate (mg to ng/well);
[2] aspirate and block for 1 hour at room temperature with 4% nonfat dry milk in PBS;
[3] aspirate and incubate 2 hours at room temperature with unbound toxin PbTx-3;
[4] aspirate and incubate 2 hours at room temperature with antibrevetoxin antibody linked to peroxidase;
[5] aspirate and add ABTS substrate and monitor change in absorbance at 405 nm.

Results:

Currently, this assay shows high nonspecific binding of antibody-peroxidase to the plates. Until we resolve the stoichiometry problem, we are uncertain if this is unaltered peroxidase binding, or antibody-peroxidase contributing to the high enzyme activity in blanks. One reason we are continuing to pursue this assay is that if unknown toxin concentrations are substituted for known toxin in step [3] above, the resulting color development in step [5] will quantify toxin in unknowns. Thus the greater color development in assays will reflect greater toxin concentrations, rather than the converse (i.e. lesser color development for greater toxin concentrations) which is characteristic of the other assays.

Synaptosome assays in microtiter plates. (figure 3, substituting synaptosomes for brevetoxin-specific IgG).

Protocol:

[1] incubate 80 μg-0.8 μg/well synaptosome preparation in Standard Binding Medium for 2 hours at room temperature;
[2] wash three times with 0.3ml PBS pH 7.4 washing buffer;
[3] block with 300 μl 1% gelatin and incubate 1 hour at room temp;
[4] rinse 3 times with 0.3ml PBS wash buffer;
[5] add toxin-urease diluted in PBS (neat to 1:200 dilution) with

1% bovine serum albumin and incubate 2 hours at 37°C;
[6] rinse 3 times with 0.3 ml distilled water;
[7] add urease reagent and bromocresol purple indicator;
[8] monitor reaction at 595 nm.

Results:

As in all assays employing the enzyme urease, some inconsistencies were noted. In general, the checkboard assays indicated that binding of toxin-urease to synaptosomes was taking place, with detectable changes occurring with synaptosome concentrations as low as 1.6 μg protein and toxin-urease dilutions of 1:20.

Future Work:

The assays were performed at a time when only toxin-urease conjugate was available. We plan to utilize synaptosomes with toxin-peroxidase and in sandwich assays using synaptosomes as primary adsorbant in the coming year. We believe the results are promising, and have begun to anticipate development of a synaptosome-toxin-antibody-peroxidase assay.

D. Summary Discussion

Table VI. Summary of Brevetoxin Microtiter Plate Assays

Adsorbant			Sensitivity[1]
Primary	Secondary	Tertiary	(ng/well)
PbTx-2	IgG α PbTx-3[2]	Protein A-urease[3]	1.0
IgG α PbTx-3[3,5]	PbTx-3-urease[4]	------	0.001
KLH-PbTx-3[4,7]	IgG α PbTx-3[4,8]	r α g IgG-peroxidase[6]	0.2
Synaptosome[4,7]	PbTx-3-urease[4,9]	------	0.2
IgG α PbTx-3	PbTx-3-peroxidase[4,9]	------	0.001
Synaptosome	PbTx-3	IgG-peroxidase α PbTx-3[4,10]	----

[1] maximum sensitivity demonstrated
[2] goat antibrevetoxin prepared by our laboratory
[3] commercial preparation
[4] brevetoxin-enzyme conjugate prepared by our laboratory
[5] keyhole limpet hemocyanin-linked brevetoxin
[6] rabbit antigoat IgG-peroxidase commercial preparation
[7] P3 fraction prepared in our laboratory
[8] brevetoxin-urease conjugate prepared by our laboratory
[9] brevetoxin-peroxidase conjugate prepared by our laboratory
[10] goat antibrevetoxin peroxidase conjugate produced by our laboratory

During the past year, we have examined several different microtiter plate assays with respect to brevetoxin detection. These are summarized above in Table VI. We have experienced mixed results in each assay explored, for a variety of reasons. We are, none-the-less, optimistic that full development will occur soon, based on the positive results we have obtained with each of the assays we have explored. The

first two assays utilize the enzyme urease, and were the first two approaches we developed.

Urease was selected for a number of reasons. Principal amongst the advantages was a general lack of urease occurrence in mammalian cells, thus theoretically reducing background activity resulting from enzyme activity in biological fluids. Second, urease is an enzyme which possesses a high turnover number, thus increasing sensitivity. However, the sensitivity of the indicator dye to pH changes and the extreme sensitivity of urease to heavy metals (both of which are expected to fluctuate in biological samples or food sources), precludes further development.

Our initial studies revealed several important factors: (i) brevetoxins alone are not good adsorbants on microtiter plates, presumably because of their proximity to surfaces and limited accessibility by antibodies to epitopes on the toxin; (ii) regardless, those antibodies which associate with bound toxin can be recognized by specific adsorbants like protein A, indicating a general applicability of sandwich type assays; (iii) brevetoxins can be successfully coupled to an enzyme without substantially reducing either the enzyme activity, or the toxin's ability to be recognized by specific antibrevetoxin antibody. Using antibrevetoxin IgG as primary adsorbant and brevetoxin-urease conjugate as enzyme probe, we observed a pg/well sensitivity; and, (iv) synaptosomes retain specific brevetoxin binding affinity in adsorbed form. Specifically, brevetoxin-urease is recognized by adsorbed synaptosomes. Thus, it appeared our only shortcoming was the enzyme we employed for detection.

Further work employing various peroxidase conjugates of both commercial and our synthetic origin indicated that our initial indications of toxin-enzyme recognition and activity were correct. Sensitivities of various peroxidase conjugates are currently evaluated at 1.0 pg/well to 200 pg/well and we expect further refinements of both blocking agent and stoichiometry to proceed in a straightforward manner in the coming year. We have also developed a toxin-KLH derivative which hydrophobically binds with high affinity to microtiter plates, and allows the epitopes on the toxin greater access to solutions of antibody applied. All assays using peroxidase are currently under further evaluation and refinement.

We expect that protein G columns used for purification of IgG classes from our goat antiserum will reduce the background levels of detectable protein not of specific IgG classes. This purification step shall precede affinity adsorption on columns containing immobilized brevetoxin, which shall yield brevetoxin specific IgG from goat antiserum. Thus, we expect our assays to be refined principally by purifying our antibody and toxin-conjugate reagents.

VI. Conclusions

[1] Antibrevetoxin antibodies can be produced in goats using brevetoxin PbTx-3 covalently linked to either bovine serum albumin or keyhole limpet hemocyanin;

[2] Antibodies induced can be purified from serum using a combination of ammonium sulfate precipitation followed by Protein G column chromatography and affinity column chromatography utilizing immobilized brevetoxin;

[3] Radioimmunoassays can be used to evaluate serum titers of antibody. These employ tritiated brevetoxins, produced by reductive tritiation;

[4] Enzyme-linked immunoassays for brevetoxins can be developed using enzyme-linked brevetoxin, enzyme-linked antibrevetoxin antibodies, enzyme-linked antigoat IgG, or enzyme-linked protein A;

[5] Immunoassays employing urease enzyme are unstable and exhibit a high degree of background activity due to nonspecific adsorption. Regardless, the assays are capable of detecting brevetoxin in the nano- to pico-gram concentration ranges;

[6] Immunoassays employing peroxidase as the probe enzyme appear more stable and reproducible, and also can be utilized in several different forms as in [4] above;

[7] Synaptosomes adhere to microtiter plates and bind brevetoxin in a specific fashion. Brevetoxin-enzyme conjugates are recognized by the binding site in synaptosomes and thus colormetric assays based on this specific binding reaction should be possible;

[8] All microtiter plate assays for the brevetoxins exhibit varying degrees of nonspecific color development.

VII. Recommendations

[1] Complete investigation of specific binding of four tritiated brevetoxin probes with goat IgG, predominantly to evaluate the specific amount of type 2 toxins bound to antibodies directed against type 1 toxins;

[2] continue to boost both goats, using KLH-brevetoxin and BSA-brevetoxin immunogens;

[3] once titers have plateaued, begin plasmaphoresis, preparing sera for IgG purification in large quantities. Purify using a combination of ammonium sulfate precipitation, Protein G affinity chromatography, and brevetoxin-affinity chromatography;

[4] re-evaluate RIAs using purified IgG from goats;

[5] continue refinement of microtiter plate assays using peroxidase enzyme, concentrating on stoichiometry and reproducibility utilizing (i) brevetoxin-peroxidase probes and each antibodies and synaptosomes, (ii) antibrevetoxin-peroxidase assays and KLH-brevetoxin;

[6] begin competitive assays and displacment assays using unbound toxin to develop standard curves;

[7] explore various biological fluids containing "spikes" of known brevetoxin to evaluate interfering materials;

[8] evaluate purified antibodies for their abilities to displace bound tritiated brevetoxin from synaptosomes.

VIII. Literature Cited

(1) Shimizu, Y., Chou, H.N., Bando, H., Van Duyne, G., Clardy, J. (1986) Structure of Brevetoxin-A (GB-1 Toxin), the Most Potent Toxin in the Florida Red Tide Organism Gymnodinium breve (Ptychodiscus brevis). JACS 108, 514.

(2) Baden, D.G., Mende, T.J., and Trainer, V.L. (1989) Derivatized brevetoxins and their use as quantitative tools in detection. In 7th IUPAC Symposium on Mycotoxins and Phycotoxins (S. Natori, Ed.) Tokyo Japan, in press.

(3) Cheng, Y.C., and Prusoff, W.H. (1973) Relationship between the inhibition constant (Ki) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. Biochem. Pharmacol. 22, 3099.

(4) Baden, D.G., Mende, T.J., Walling, J., Schultz, D. (1984) Specific Antibodies Directed Against Toxins of Ptychodiscus brevis (Florida's Red Tide Dinoflagellate). Toxicon 22,783.

(5) Poli, M.A., Mende, T.J., Baden, D.G. (1986) Brevetoxins, Unique Activators of Voltage-Sensitive Sodium Channels, Bind to Specific Sites in Rat Brain Synaptosomes. Mol. Pharmacol. 30, 129.

(6) Baden, D.G., Mende, T.J., Szmant, A.M., Trainer, V.L., Edwards, R.E., Roszell, L.E. (1988) Brevetoxin Binding: Molecular Pharmacology Versus Immunoassay. Toxicon 26, 97.

(7) Scharpe, S.L., Cooreman, W.M., Blomme, W.J., Laekman, G.M.(1976) Clinical Chem. 22, 733.

(8) Wisdom, G.B.(1976) Clinical Chem. 22, 1243.

(9) Baden, D.G. (1987) Binding assays for the quantitative detection of P. brevis polyether neurotoxins in biological samples and antibodies as therapeutic aids for polyether marine intoxication. Annual Summary Report DAMD17-87-C-7001, U.S. Army Research and Development Command, Fort Detrick, Frederick, Maryland, 31 pp.

(10) Dodd, P.R., Hardy, J.A., Oakley, A.E., Edwardson, J.A., Perry, E.K., Delaunoy, J.P. (1981) A Rapid Method for Preparing Synaptosomes: Comparison with Alternate Procedures. Brain Research 226, 107.

(11) Bradford, M.M.(1976) A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein. Analyt. Biochem. 72, 248.

(12) Abraham, G.E., and Grover, P.K. (1971) Covalent Linkage of Steroid Hormones to Protein Carriers for use in Radioimmunoassay. In Competitive Protein Binding Assays (W.Odell, W. Daughaday, eds.) J.B. Lippincott, Philadelphia, 140.

(13) Bigazzi, P., Wicker, K., Gorzyski, E., Zeschke, R., Puleo, J., Andres, G., Gutcho, S. (1973) Procedures Using Labeled Antibodies or Antigens. In Methods in Immunodiagnosis (N.R. Rose, P.E. Bigazzi, eds.) John Wiley, New York, 107.

(14) Baden, D.G. and Mende, T.J. (1988) Characterization of the P. brevis polyether neurotoxin binding component in excitable membranes. Final Report DAMD17-85-C-5171, U.S. Army Medical Research and Development Command, Fort Detrick, Frederick Maryland, 16 pp.

(15) Edwards, R.A.(1988) Turtle brain synaptosomes: preparation and brevetoxin binding. M.S. thesis, University of Miami, 79 pp.

(16) Catterall, W.A. and M.A. Risk (1981) Toxin T46 from *Ptychodiscus brevis* (formerly *Gymnodinium breve*) enhances activation of voltage-sensitive sodium channels by veratridine. Mol. Pharmacol. 19, 345.

(17) Catterall, W.A., and Gainer, M. (1985) Interaction of brevetoxin-A with a new receptor on the sodium channel. Toxicon 23, 497.

(18) Tijssen, P. (1985) Practice and Theory of Enzymatic Immunoassays. Elsevier Science Publishers, Amsterdam, 549 pp.

DISTRIBUTION LIST

5 copies	Commander US Army Medical Research Institute of Infectious Diseases ATTN: SGRD-UIZ-E Fort Detrick, Frederick, Maryland 21701-5011
1 copy	Commander US Army Medical Research and Development Command ATTN: SGRD-RMS-RMI-S Fort Detrick, Frederick, Maryland 21701-5012
1 copy	Dean School of Medicine Uniformed Services University of the Health Sciences 4301 Jones Bridge Road Bethesda, MD. 20814-4799
1 copy	Commandant Academy of Health Sciences, US Army ATTN: AHS-CDM Fort Sam Houston, TX 78234-6100
2 copies	Defense Technical Information Center (DTIC) ATTN: DTIC-DDAC Cameron Station Alexandria, Virginia 22304-6145